

IMPROVED TECHNIQUES FOR THE PREPARATION OF POLYTENE CHROMOSOMES FOR SOME ANOPHELINE MOSQUITOES¹

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ABSTRACT. Improvements in the techniques for observation and preparation of polytene chromosomes from anopheline mosquitoes are reported. The improvements are as follows: an observation method for polytene chromosomes using both phase-contrast and interference microscopy with the same microscope; an improved polytene chromosome preparation technique, a field collection

method and a rearing method for obtaining excellent material for cytogenetic research and investigation. The described methods were successful for certain species complexes for which poor results were obtained using previously described methods. These techniques are useful not only for laboratory research but also for field investigations and the cytotaxonomical identifications of malaria vectors.

During the past 20 years there have been significant advances in cytogenetic studies of the genus *Anopheles*, and many contributions have been made in the cytotaxonomy of sibling species, with particular reference to vectors of disease. Coluzzi and Kitzmiller (1975) have listed the anopheline species whose polytene chromosomes have been described in

detail and White et al. (1975, WHO, unpublished) have prepared a bibliography of illustrations and maps of anopheline polytene chromosomes. Such chromosomal studies employed the polytene chromosome preparation techniques for Diptera as described by Nicoletti (1959). However, the original or slightly modified techniques (French et al. 1962, Kanda 1971) cannot be successfully applied to all species of *Anopheles*, *Drosophila* or *Chironomus*. There remain many complexes or species groups of *Anopheles*, containing sibling species, for which maps have not been produced.

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Such complexes include the *An. punctulatus* complex, the *An. hyrcanus* complex, the *An. leucosphyrus* complex, the *An. pyretophorus* group and the *An. minimus* complex etc. which are found mainly in East Asia.

The above mentioned sibling species complexes appear to have quite different chromosomal characteristics from those of the *An. gambiae* complex, the *An. maculipennis* complex and the *An. punctipennis* complex and it is often quite difficult to recognize and confirm the banding patterns of the polytene chromosomes. This is due to chromosomal distortions such as shrinkage, looping, rolling and twisting caused by the fixatives used in preparation techniques. In order to rectify this situation the original chromosome preparation technique and the methods of observation have been significantly improved resulting in successful application in both the laboratory and the field.

COLLECTION OF ANOPHELINE MOSQUITOES IN THE FIELD

For the collection of adult female mosquitoes from field locations the bait net trap technique is recommended. A variety of baits (human and animals) may be used and the fully blood-fed mosquitoes are easily collected from the sides of the net. This method of capture is efficient in collecting sufficient numbers at one time. Batches of female mosquitoes collected from different baits and environmental locations (indoor/outdoor house collections) should be kept separate from each other. Within any one batch, the specimens should be identified according to known morphological criteria and separated, placing 20-25 individuals into a paper cup and holding them for 2-3 days, at 25°C and sufficient humidity, until gravid. The gravid females of each species should then be separated, transferring each individual female to a small flat bottomed glass tube (17 mm dia. by 50 mm high) which has a piece of damp filter paper on the bottom. The damp

filter paper serves as an egg laying surface and maintains the required humidity inside the tube. The tubes must be checked regularly until the females have oviposited.

LABORATORY REARING OF ANOPHELINE MOSQUITOES FOR CHROMOSOME MATERIAL. At the laboratory the individual egg batches from the field are removed from the glass tubes, the number of eggs per batch recorded and a sample of eggs removed for morphological study. The bulk of the eggs from each batch should then be placed into small bowls, approximately 90 mm in diameter, containing deionized water. After hatching, the young larvae are then transferred to rearing trays each containing about 500 ml of deionized water and pieces of the tropical garden grass *Axonopus compressus* (or similar species). The root system of this plant is very important for maintaining clean water for the rearing of healthy larvae and pupae so care should be taken when uprooting and cleaning the plant before placing in the rearing trays. A suitable size for the rearing trays is approximately 350 mm long × 240 mm wide × 60 mm deep.

To insure the healthy and normal development of the larval stages 2 specially prepared food mixtures are required. The first recipe, for 1st and 2nd instar larvae, consists of even parts of oatmeal, dried yeast, wheat germ and cornstarch or Farex. The 2nd recipe, for 3rd and 4th instar larvae, consists of 1 part of the following mixture to be added to the 1st recipe: 750g Nestrum or Lactogen; 250g cornstarch or Farex; 50 g ox liver powder; 15 mg vitamin B complex. These mixtures should be ground as finely as possible during preparation so that the larval food will spread evenly and float over the surface of the water. Control in applying the correct amount of larval food can be obtained by using a domestic salt shaker. It is important that only sufficient larval food be given since excessive amounts will cause cloudy water and scum formation. The young larval stages should be fed once per day and the older stages twice per day.

Throughout the rearing of the aquatic stages the water should be maintained in a clean state and be free of other organisms which might otherwise disturb larval development. By following the above procedures excellent chromosome preparations can be obtained from the healthy larvae.

PROCEDURES FOR SALIVARY GLAND DISSECTION AND STAINING OF POLYTENE CHROMOSOMES. Two glass cavity slides are placed on the dissection plate of a dissecting microscope, one cavity containing 15% acetic acid and the second cavity containing 1% sodium citrate. The early 4th instar larval specimen to be dissected should first be blotted with filter paper to remove excess water and then placed into the cavity containing 15% acetic acid. To open the thorax, a fine dissection needle is inserted into the dorsal aspect of the thorax directly under the cuticle. A second fine dissection needle, placed on the outer surface of the cuticle, is then rubbed against the first needle so as to split the cuticle in an anterior-posterior direction along the dorsal surface of the thorax. The larval head is then severed from the body and the pharynx and the esophagus pulled out to expose the salivary gland tissues within the thorax. The whole larval body is then removed and placed into the 2nd cavity containing 1% sodium citrate where the white colored anterior lobe of the salivary gland body is carefully separated from the translucent posterior lobe. The anterior lobe is then transferred into 1/2 drop of 45% acetic acid, on a siliconized coverslip, for fixation. The posterior lobe is discarded.

Where chromosomal shrinking is known to occur the following alternative fixatives may be used: (a) even parts of 45% acetic acid and lactic acid or (b) after fixation in 45% acetic acid for a short period add 1/2 droplet of acetolactic acid solution and mix. The fixation method to be followed will depend upon the anopheline species under investigation. Although 45% acetic acid had proved to be an excellent fixative for many species of anopheline mosquitoes, some excep-

tions have been found in the *An. punctulatus* complex, the *An. minimus* complex and the *An. aitheni* species group. The fixation time using 45% acetic acid can be determined by the observed degree of shrinking of the polytene chromosomes.

The stain is prepared by mixing 1 part of concentrated orcein stain with 3 parts of lacto-acetic acid (1:1). A 1/6 droplet of prepared orcein stain is then mixed with the fixative on the siliconized coverslip and the tissues are left undisturbed for a period of 1-2 min. A scrupulously clean glass microscope slide is then lowered onto the droplet so that the siliconized coverslip will adhere to the glass slide by capillarity and at the same time slightly flatten the tissues. The slide is then turned over, so the coverslip now lies uppermost, and firmly wrapped in absorbent paper. The coverslip should then be tapped gently several times with a finger to squash the salivary gland tissue and spread the polytene chromosomes. The status of the chromosome preparation may then be determined by examination under a phase-contrast microscope. Examination by interference microscopy can also be carried out if desired.

In order to preserve the squash preparation and prevent it from drying out a semi-permanent preparation can be made by sealing the edges of the coverslip with transparent nail varnish. Such preparations, if kept in a deep freeze unit at -20°C , will remain in a satisfactory condition for a period of one year or more.

PREPARATION OF PERMANENT MOUNTS. Permanent chromosome preparations can be prepared following the method of Conger and Fairchild. However, since liquid nitrogen may not be readily available in many tropical countries, solid carbon dioxide (dry ice) has been found to be a suitable substitute.

The squash preparation should first be frozen by dipping into liquid nitrogen contained in a wide mouthed vacuum flask. The siliconized coverslip can then be 'flicked' off using a safety razor blade leaving the salivary glands adhering to

the glass slide. The preparation is then dehydrated by passing through 4-5 changes of alcohols, ranging from 70% to absolute alcohol in covered glass staining jars. After dehydration the preparation is removed from the final jar of absolute alcohol but prior to the tissue drying out mounting medium (Einschlussmittel L-15) is placed on the preparation and covered with a clean coverslip. Such preparations are then initially dried out at room temperature for 24 hr and then placed in a drying oven at 45-48°C for 24 hr or more.

MICROSCOPIC EXAMINATION. The morphological banding characters of polytene chromosomes can be studied when viewed through the interference microscope by changing the direction of polarized light from 0° to 90°. In this way the configurations of the banding patterns, as observed by phase-contrast and interference microscopy, can be compared in the same preparation when the 2 optical systems are combined on the same microscope. The author is presently using the Nikon interference microscope type XF-NT-21. Utilizing these 2 optical systems staining characteristics (as viewed by phase-contrast microscopy) and the chromosomal characteristics, due to the illumination of the chromosomal component by polarized light (as viewed by interference microscopy) the same bands can be recognized. Through such comparative studies knowledge may be gained regarding the relationships between the banding configurations of polytene chromosomes.

RESULTS OF THE APPLICATION OF THE IMPROVED TECHNIQUES ON SOME ANOPHELINE MOSQUITOES

Good polytene chromosome preparations have been made from field material of some member species of the *An. hyrcanus* complex. In particular, *An. crawfordi* gave excellent chromosome preparations yielding recognizable banding pattern configurations when 45% acetic

acid was used for fixation. In other member species of this complex the chromosome complements shrank and became hard due to the strong action of the fixative. The application of the improved techniques to the anopheline complexes of *An. punctulatus*, *An. minimus*, *An. leucosphyrus* and *An. pyretophorus* has proved successful, and similar results to those mentioned above have been obtained.

Comparative studies on the chromosomal configuration in polytene chromosomes, as viewed under phase-contrast and interference microscopy, have shown the existence of significant morphological detail on the surfaces of chromosome complements. The stained chromosomes, when viewed through the phase-contrast microscope, show the staining characteristics of the banding patterns. When viewed through the interference microscope, the surface characteristics of the chromosomes are revealed in a way not unlike results from the scanning electron microscope. These studies have shown that the chromosomal surface characters, such as banding, dots, puffs, constrictions, roughness and smoothness, do not necessarily coincide with the staining characters at the same locus of the chromosome, as shown in Figures 1, 2 and 3. Since it is held that the chromosomal surface characteristics will yield more reliable and exact genetic information than the staining characters alone, microscopic examination of polytene chromosomes should be made using both interference and phase-contrast microscopy.

DISCUSSION ON THE APPLICATION OF THE IMPROVED TECHNIQUES

The reasons necessitating the development of an improved technique for the preparation of polytene chromosomes have been explained elsewhere in the text. Trials have shown that the chemical formulation of the fixatives is crucial for the preparation of chromosomes with recognizable characters.

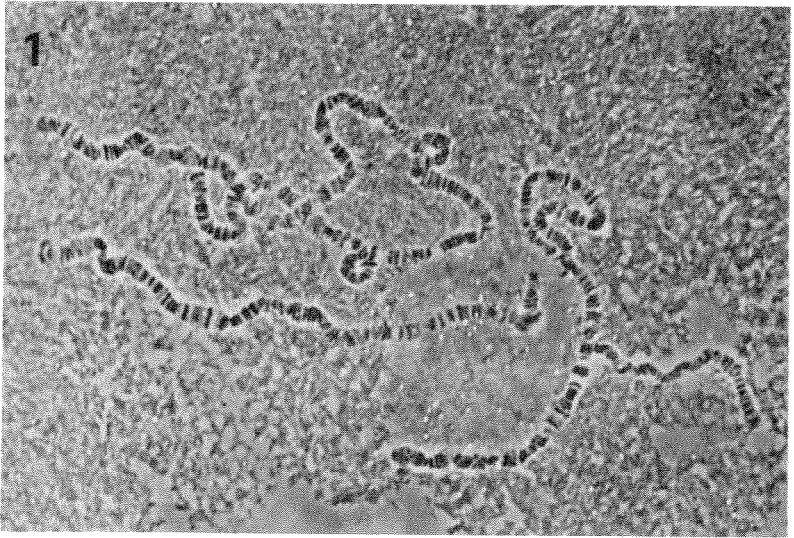


Fig. 1. The structure of polytene chromosomes of a member of the *A. punctulatus* by phase-contrast photography.

Sharma and Sharma (1965), concluded that acetic acid precipitates nucleic acid and dissolves histones but is incapable of fixing cytoplasmic protein. The main use of acetic acid in the study of chromosome structure is to prevent shrinkage of the chromosomes and preserve their structure without distortion. The chemicals used in Carnoy's fixative, in combination with acetic acid or others, are not effective for chromatin and have the property of rapid penetration. Mercuric chloride precipitates protein very strongly, and chloroform, which is generally used in fixatives, dissolves the outer fatty and waxy secretions to facilitate the penetration of the fixative. Thus acetic acid was found to be the most suitable chemical for fixation for the preparation of chromosomes.

In the initial stage of the preparation schedule, light fixation of the chromosomes is achieved using 15% acetic acid as this serves to retain the formation and structure of the chromosomes without causing shrinkage. Since shrinkage due to acetic acid found in the anopheline spe-

cies mentioned above and some species of *Chironomus*, these pretreatments were incorporated before the fixation by 45% acetic acid. Removal of the salivary gland tissues in a 1% solution of sodium citrate retards the fixation process and keeps the chromosomes soft without any shrinkage or hardening. With final fixation in 45% acetic acid, the chromosomes can be spread easily with only light, gentle tapping on the siliconized coverslip. The chromosomes are then visible without any shrinkage, twisting, rolling or approximation of bands. In order not to conceal the surface characteristics of the chromosomes for observation by interference microscopy, the preparations should be stained with orcein stain so that the banding patterns may be observed through the phase-contrast microscope. The uptake of the stain can be halted by placing the temporary preparations in a freezer unit, at approximately -20°C , after a short period at room temperature.

Comparative studies of the banding patterns and surface structures and configurations at the same loci of the

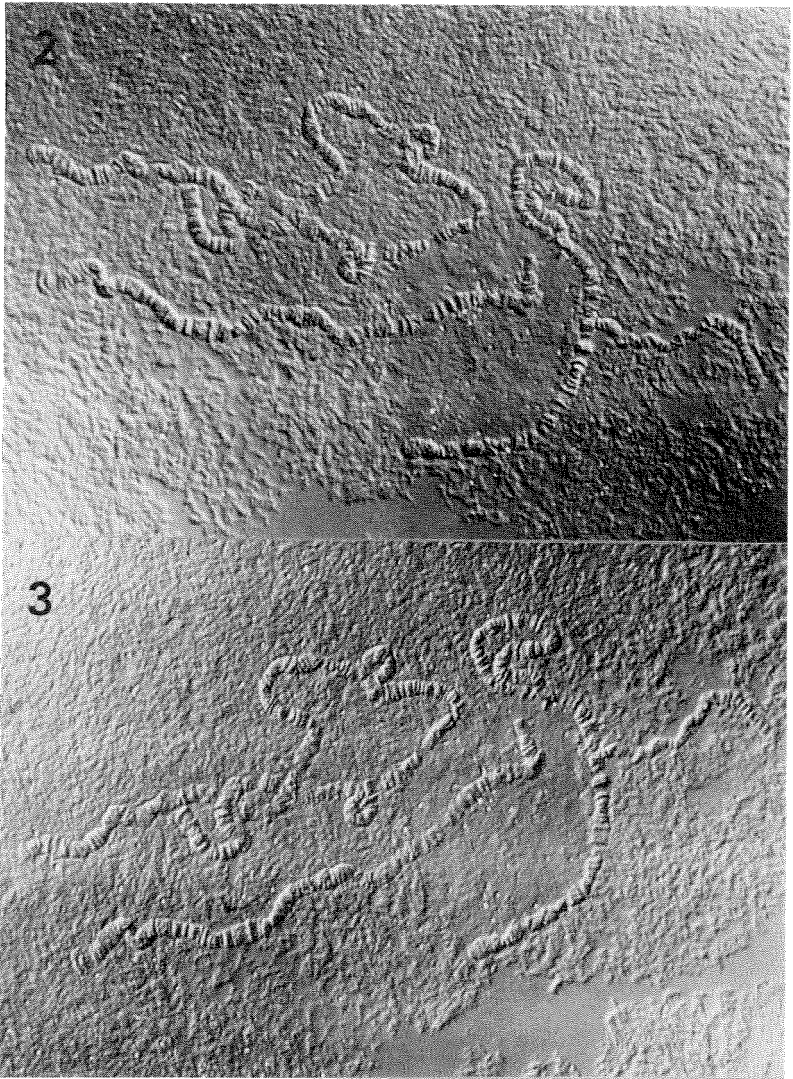


Fig. 2 and 3. The structures of the same complement using interference microscopy. Two sources of polarized light at a difference of 90° from each other are employed.

In these figures the banding patterns and surface configurations may be compared at each locus. Staining patterns do not always parallel configurations observed by interference microscopy.

chromosomes can be achieved if the two optical systems are combined on the same microscope. Such observations will give more exact information on chromosomal morphology than phase-contrast microscopy alone.

Healthy larvae collected in nature generally give excellent preparations of salivary gland polytene chromosomes but equally as good preparations can be obtained from artificially reared larvae provided that the correct rearing method is used. The previously described method has given excellent results for many anopheline species collected in the Pacific and Asian areas.

When mosquito material is being used for taxonomic studies in which cytogenetic, morphological and biological characters are to be used for the identification of sibling or sympatrically occurring species, it is important to consider all the stages in the life-cycle. Thus from the egg batches of single female samples of eggs-larvae-pupae-adults (males and females) should be preserved for examination. To obtain good egg batches initially, the bait net trap has been found to be the most suitable capture method for fully blood-fed female mosquitoes.

All the above mentioned techniques are practical for field investigations in vector control as well as being important to genetic research in anopheline mosquitoes.

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